Pertussis toxin catalyzes the ADP-ribosylation of two distinct peptides, 40 and 41 kDa, in rat fat cell membranes

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Pertussis toxin catalyzes the ADP-ribosylation of a single 41-kDa peptide of membranes prepared from rat hepatocytes, S49 mouse lymphoma wild-type and *cyc*-mutant cells. This 41-kDa peptide has been shown to be the α-subunit of the inhibitory, guanine nucleotide binding regulatory component of adenylate cyclase (N_i). Incubating membranes of rat fat cells with pertussis toxin and [^{32}P]NAD^+ radiolabels a 41- and a 40-kDa peptide. Possible homologies between these peptides were investigated by comparing the electrophoretic patterns of proteolytic fragments derived from each of them that are radiolabeled by [^{32}P]NAD^+ and pertussis toxin. The 40-kDa substrate for pertussis toxin-catalyzed ADP-ribosylation and the α-subunit of N_i in rat fat cells appear to be homologous, but non-identical peptides.

Pertussis toxin *ADP-ribosylation* *Rat fat cell* *Inhibitory guanine nucleotide-binding regulatory component*

1. INTRODUCTION

The toxin of *Bordetella pertussis,* also called islet-activating protein, attenuates receptor-mediated inhibition of adenylate cyclase [1–5] and catalyzes the ADP-ribosylation of a protein that both binds guanine nucleotides and is involved in inhibitory regulation of adenylate cyclase [6–9]. This multimeric protein has recently been purified from liver [10] and human erythrocytes [11] and is the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase, referred to as N_i [12]. The subunit of N_i that is a substrate for pertussis toxin-catalyzed ADP-ribosylation appears to be a single 41-kDa peptide in a variety of cell types [5–11]. Pertussis toxin has been shown to attenuate inhibition of cyclic AMP accumulation in rat cells by adenosine, to increase the stimulatory effects of insulin on fat cell metabolism, and to interfere with α_1-adrenergic stimulation of phosphatidylinositol turnover [13]. Here, the substrates for pertussis toxin-catalyzed ADP-ribosylation in membranes from rat fat cells are investigated. Two peptides, 40 and 41 kDa, were specifically labeled in membranes incubated with activated pertussis toxin and [^{32}P]NAD^+. These substrates are distinct but structurally homologous peptides. The 40-kDa peptide may be involved in receptor-mediated actions of hormones that are not coupled to adenylate cyclase.

2. EXPERIMENTAL

Fat cells were isolated from 150 g fed, female Sprague-Dawley (SD strain) rats by the method of [14]. Membranes were prepared as in [15]. Membranes (0.1 mg protein) were incubated for 30 min at 30°C in the presence of 250 mM KH_2PO_4 (pH 7.5), 5 mM $MgCl_2$, 10 mM arginine, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, GTP-γS or GDP-βS, 2 mM dithiothreitol, 20 mM urea, bovine serum albumin (0.1 mg/ml), and pertussis toxin (10–100 μg/ml) that was activated with 20 mM dithiothreitol for 10 min at 37°C. The membranes were washed, solubilized, subjected to polyacrylamide gel electrophoresis, and autoradiography as

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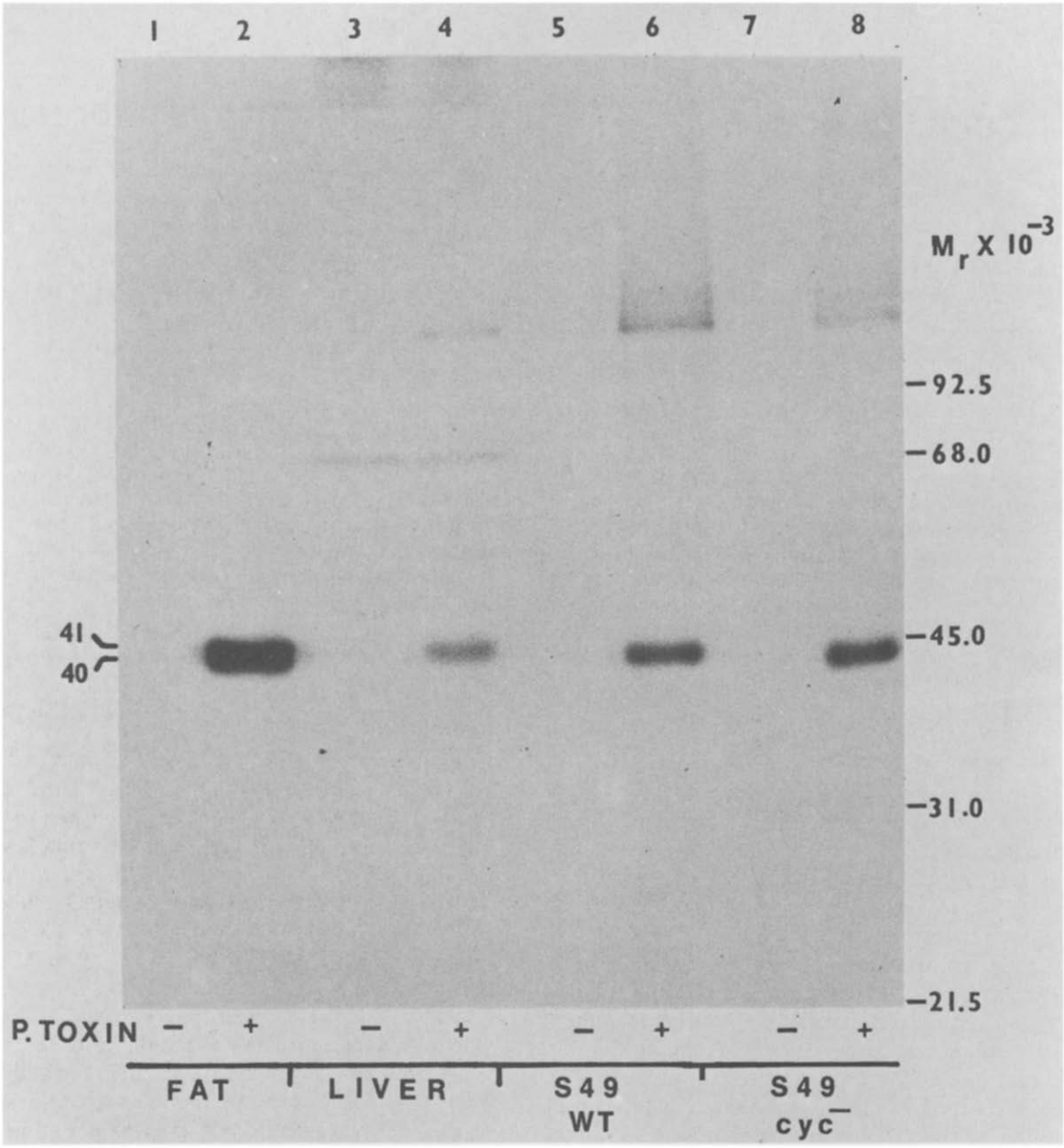

Fig.1. Autoradiogram of polyacrylamide gel analysis of membrane proteins labeled in the presence of pertussis toxin and [^{32}P]NAD$^+$. Membranes prepared from rat fat cells, rat hepatocytes, and S49 mouse lymphoma wild-type and *cyc*$^-$ mutant cells were incubated for 30 min at 30°C with activated pertussis toxin (0.1 mg/ml) and [^{32}P]NAD$^+$ (0.1 μM, 10 μCi). Gel electrophoresis and autoradiography were performed as described in section 2. The autoradiogram is a 2-day exposure.

in [16]. S49 mouse lymphoma wild-type and *cyc*⁻ mutant cells were grown and their membranes prepared as in [16]. Membranes were prepared from rat hepatocytes [17]. Peptide maps of substrates for pertussis-toxin catalyzed ADP-ribosylation were generated as described previously [18]. Pertussis toxin was purified approx. 1800-fold for use in these studies [19].

3. RESULTS AND DISCUSSION

The substrates for pertussis toxin-catalyzed ADP-ribosylation were identified as follows: membranes from rat fat cells, rat hepatocytes, S49 mouse lymphoma wild-type and *cyc*⁻ mutant cells were incubated with activated pertussis toxin and [^{32}P]NAD$^+$, the membrane proteins were separated on polyacrylamide gels by electrophoresis in SDS, and the radiolabeled products identified by autoradiography (fig.1). A 41-kDa membrane protein of liver cells (lane 4), S49 wild-type cells (lane 6), and S49 *cyc*⁻ mutant (lane 8) was the predominant substrate for pertussis toxin-catalyzed ADP-ribosylation. Fat cells also display a 41-kDa membrane peptide that is specifically labeled in the presence of [^{32}P]NAD$^+$ and pertussis toxin (fig.1, lane 2). However, in sharp contrast to the single 41-kDa peptide observed in liver and S49 cell membranes, in fat cells an additional membrane peptide, of 40-kDa, is also ADP-ribosylated by [^{32}P]NAD$^+$ in the presence of pertussis toxin. The 40-kDa peptide does not appear to be a degradation product of the 41-kDa peptide because (i) the membranes are freshly prepared from cells in the presence of a variety of protease inhibitors [20], (ii) fat cell ghosts prepared by hypotonic lysis display both peptides, and (iii) the amount of each peptide relative to the other remains constant and is not altered by incubation at 22°C or exposure to freeze-thaw cycles (not shown).

Cholera toxin catalyzes the ADP-ribosylation of subunits of the stimulatory, guanine nucleotide-binding regulatory component of adenylate cyclase [16,21–23], referred to as N_s [12]. The 41-kDa substrate for pertussis toxin-catalyzed labeling is observed in membranes of S49 *cyc*⁻ cells [6], which do not contain N_s [24–27]. ADP-ribosylation of fat cell membranes by both toxins clearly distinguishes the substrates for pertussis toxin-catalyzed ADP-ribosylation (40- and 41-kDa peptides) from the

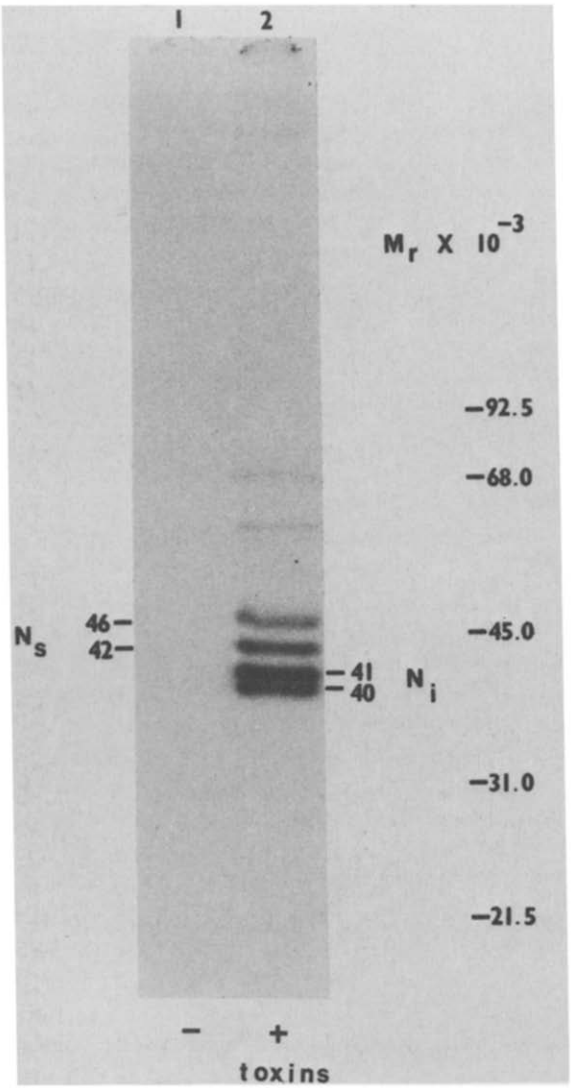

Fig.2. Autoradiogram of polyacrylamide gel analysis of membrane proteins labeled in the presence of bacterial toxins and [^{32}P]NAD$^+$. Fat cell membranes were incubated with [^{32}P]NAD$^+$ and vehicle (lane 1) or cholera toxin (0.1 mg/ml) and pertussis toxin (0.1 mg/ml) together as described in the legend to fig.1. Both toxins were activated with 20 mM dithiothreitol for 10 min at 37°C. The autoradiogram is a 2-day exposure.

subunits of N_s that are cholera toxin targets (42- and 46-kDa peptides) (fig.2). The guanine nucleotide specificity of the bacterial toxin-catalyzed ADP-ribosylation reaction also differentiates the substrates of pertussis toxin from those of cholera toxin (fig.3). GTP and GTP-γS enhance, while

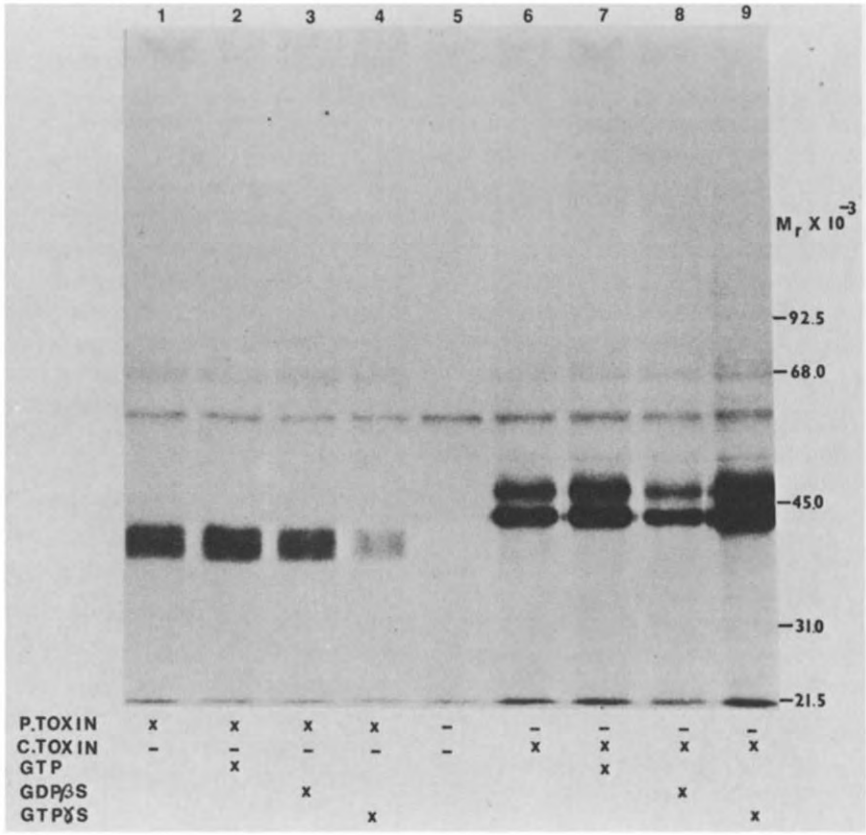

Fig.3. Effects of guanine nucleotides on bacterial toxin-catalyzed ADP-ribosylation of N_s and N_i by [^{32}P]NAD$^+$. Fat cell membranes were incubated with the indicated guanine nucleotide (0.1 mM) for 30 min on ice and then incubated for 30 min at 30°C with [^{32}P]NAD$^+$ and vehicle (lane 5) or activated pertussis toxin (lanes 1–4) or cholera toxin (lanes 6–9). For details see legend to fig.1. This autoradiogram is a 3-day exposure.

GTP-βS reduces the amount of labeling catalyzed by cholera toxin into the subunits of N_s (lanes 6–9). The amount of labeling catalyzed by pertussis toxin into both the 40- and 41-kDa peptides of the fat cell membranes is greater in the presence of GDP-βS as compared to GTP-γS (lanes 1–4).

Possible homologies between the 40- and 41-kDa peptides of rat fat cell membranes were investigated by comparison of the electrophoretic patterns of proteolytic fragments derived from each of these peptides. Membranes of rat fat cells were radiolabeled by [^{32}P]NAD$^+$ in the presence of pertussis toxin. The 40- and 41-kDa peptides were separated electrophoretically on 20-cm tube 10% acrylamide gels (1st dimension). The labeled peptides were then treated with either elastase, α-chymotrypsin, or *S. aureus* V8 protease and subjected to electrophoresis on a 14% acrylamide slab gel (2nd dimension). The autoradiograms of the partial proteolytic peptide maps are shown in fig.4. Although several fragments generated from the 40- and 41-kDa peptides upon partial proteolysis by elastase were common to both, it is clear that the peptide maps are non-identical (fig.4, panel A). The similarities between the fragments generated from the 40- as compared to the 41-kDa peptides by partial digestion with α-chymotrypsin are striking (fig.4, panel B). Several fragments unique to the 40- or 41-kDa peptides are also generated by α-chymotrypsin. Peptide maps of partial digests by V8 protease likewise display not only fragments common to both pertussis toxin targets, but several fragments unique to only one of these peptides (not shown). The similarities between peptide

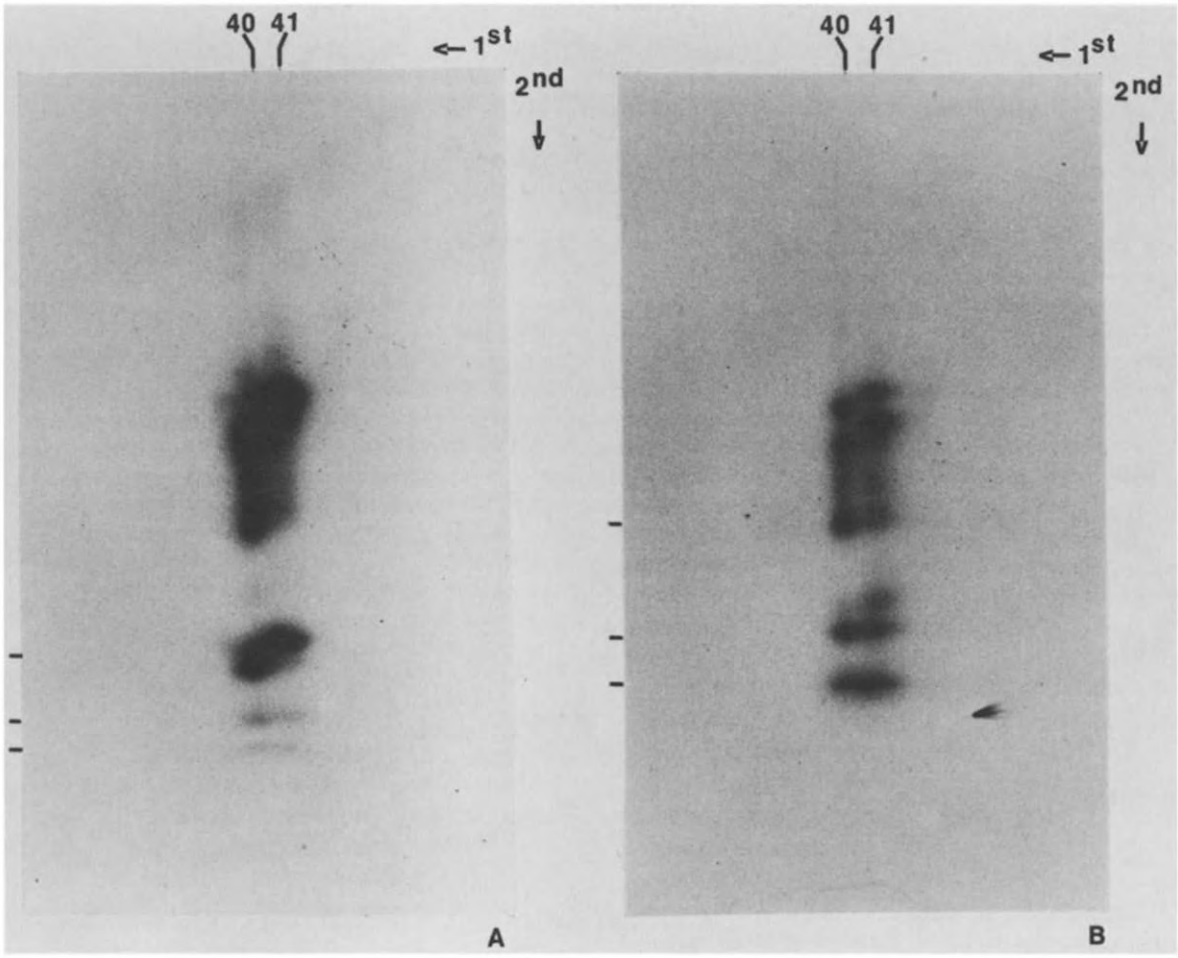

Fig.4. Autoradiogram of polyacrylamide gels prepared from partial proteolytic digests of both the 40 and 41-kDa peptides of rat fat cells that had been radiolabeled in the presence of pertussis toxin and [^{32}P]NAD$^+$. Membranes were prepared as described in the legend to fig.1. The labeled membranes were solubilized in SDS and subjected to electrophoresis on 10% polyacrylamide tube gels in the first dimension. The first dimension tube gel was then bonded to a 5% stacking gel using agarose containing either 8 μg of elastase (panel A) or 50 μg of α-chymotrypsin (panel B). The samples were moved into the stacking gel by electrophoresis and the current was then turned off for 30 min to allow partial proteolysis of the peptides. The proteolytic fragments were separated in the second dimension by electrophoresis in the presence of SDS on a 14% polyacrylamide gel. Autoradiography was performed for 10 days. Peptides common to both digests are indicated by horizontal lines.

maps of partial digests of 40- and 41-kDa peptides of rat fat cell membranes labeled with [^{32}P]NAD$^+$ and pertussis toxin suggest that these toxin substrates are homologous, but non-identical proteins.

The 41-kDa substrate for pertussis toxin-catalyzed ADP-ribosylation is present in all mammalian tissues that we have examined to date and appears to be the α-subunit of N_i [7–11]. We have observed the 40- in addition to the 41-kDa substrate in membranes of rat fat cells (present study) and rabbit heart (not shown). The role of the 40-kDa peptide substrate for pertussis toxin-catalyzed ADP-ribosylation remains to be elucidated. The ability of pertussis toxin to interfere with α_1-adrenergic stimulation of phosphatidylinositol turnover and

to augment effects of insulin on lipolysis [13] raises the possibility that the 40-kDa peptide that is homologous to the α-subunit of N_i may be an N protein that is coupled to effector systems other than adenylate cyclase. These interesting possibilities are currently under investigation.

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